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The Challenge of Studying TiO_2 Nanoparticle Bioaccumulation at Environmental Concentrations: Crucial Use of a Stable Isotope Tracer

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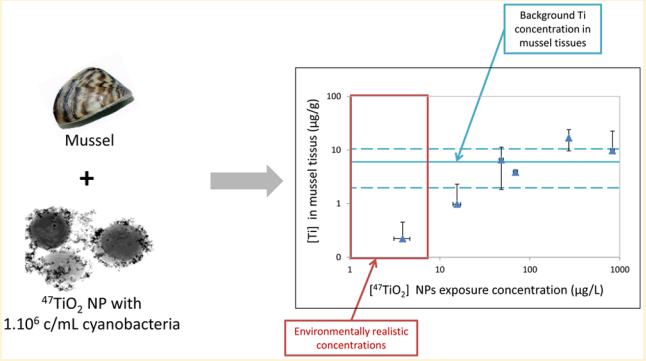
Supporting Information

ABSTRACT: The ecotoxicity of nanoparticles (NPs) is a growing area of research with many challenges ahead. To be relevant, laboratory experiments must be performed with well-controlled and environmentally realistic (i.e., low) exposure doses. Moreover, when focusing on the intensively manufactured titanium dioxide (TiO_2) NPs, sample preparations and chemical analysis are critical steps to meaningfully assay NP's bioaccumulation. To deal with these imperatives, we synthesized for the first time TiO_2 NPs labeled with the stable isotope ^{47}Ti. Thanks to the ^{47}Ti labeling, we could detect the bioaccumulation of NPs in zebra mussels (*Dreissena polymorpha*) exposed for 1 h at environmental concentrations via water (7−120 μg/L of $^{47}TiO_2$ NPs) and via their food (4−830 μg/L of $^{47}TiO_2$ NPs mixed with 1×10^6 cells/mL of cyanobacteria) despite the high natural Ti background, which varied in individual mussels. The assimilation efficiency (AE) of TiO_2 NPs by mussels from their diet was very low (AE = 3.0 ± 2.7%) suggesting that NPs are mainly captured in mussel gut, with little penetration in their internal organs. Thus, our methodology is particularly relevant in predicting NP's bioaccumulation and investigating the factors influencing their toxicokinetics in conditions mimicking real environments.



INTRODUCTION

The number of manufactured products containing industrial titanium dioxide nanoparticles (TiO_2 NPs) is tremendously increasing including paints, coatings, sunscreens, food colorings and toothpastes[1] which inevitably end up in aquatic ecosystems. This rapid rise makes it important to establish the environmental and potential health impacts of TiO_2 NPs in realistic conditions. It is generally assumed that the toxicity of NPs depends on their size, shape, and crystalline phase, and is further impacted by the surrounding media,[2] though the key parameters controlling their toxicity are not clearly identified. The literature may be very controversial due to a lack of homogeneity and quality in the studies' methodology.[3] Before addressing how NPs might cause harm in ways that are not readily understood at the moment, we need first to carefully investigate their uptake route and bioaccumulation mechanisms.

The investigation of bioaccumulation mechanisms must be conducted at environmentally realistic NP's concentrations to be accurately extrapolated to the environment. Previous studies of NP's bioaccumulation, including TiO_2 NPs, were conducted with acute exposure concentrations, (usually 1−10 mg/L) strongly exceeding the TiO_2 concentrations predicted for manufactured NPs in surface waters, which in Europe occurs at μg/L levels or less.[4] Thus, environmentally realistic low concentrations of manufactured NPs remained poorly tested. This is also due to the complexity of discriminating newly acquired Ti from the natural high background levels of Ti occurring in biological organisms. These natural Ti backgrounds, which are expected to be in the range 1−50 μg/g dry weight,[5,6] arise from the natural abundance of TiO_2 particles (they occur at 80 μg/L in the Seine river[7] while the estimated contribution of manufactured TiO_2 NPs is only about 1 μg/L[4]). To overcome these problems, one can label NPs with stable isotopes. Stable isotope tracers have already been used to investigate metal uptake[8] and were recently applied to NPs.[9−13] Gulson et al.[12] and Dybowska et al.[9] used for the first time this approach to study, respectively the absorption of the ZnO NPs present in a sunscreen, and to detect the bioaccumulation of

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ZnO NPs in freshwater snails exposed to environmentally realistic concentrations.

Up to now, the stable isotope method was not applied to TiO_2 NPs, mainly because the quantification of low concentrations of Ti is complex at the level of both sample preparation and Ti detection with inductively coupled plasma mass spectrometry (ICP-MS). Digestion of TiO_2 NPs samples to completely dissolve the elements prior to their analysis is problematic due to the refractory nature of TiO_2. Existing literature related to TiO_2 dissolution compares alkaline fusion methods with acidic digestion, in an open hot-plate or with microwave heating.[14] A mixture of acids including hydrofluoric acid (HF) is usually used, although HF is unsafe. Fusion procedures are especially well suited to dissolve TiO_2 NPs[15,16] but they are often discarded due to the large quantity of salts they introduce in the final solution, which can interfere during ICP-MS analysis. However, the new generation of ICP-MS equipped with collisional kinetic energy discrimination (KED) could reduce interferences thanks to a low mass cutoff mode and allow an accurate measurement of low Ti concentration in these types of matrices.

In the present study, we synthesized isotopically enriched TiO_2 NPs ($^{47}TiO_2$ NPs) to quantify Ti bioaccumulation in zebra mussels exposed at low environmental concentrations, in the presence or absence of their typical food (the cyanobacterium *Synechocystis*). The choice of zebra mussels was motivated by their wide distribution and abundance in freshwater biotopes, thereby making these organisms suitable for future NP's biomonitoring.[17] With zebra mussels we can thoroughly study NP's accumulation (considering both uptake and excretion), while in future investigations, with the wealth of *Synechocystis* mutants, we will investigate the influence of particular characteristics of this environmentally important organism (thanks to their powerful photosynthesis, cyanobacteria make up a large part of the oxygen and biomass for the food chain). For instance, it will be possible to study the influence of cyanobacterial exopolysaccharides on NP's uptake by mussels and other organisms with higher trophic levels in the food chain (fish and arthropods). In the present study, a particular attention was paid to the stability of NPs during exposures. Indeed, since TiO_2 NPs tends to aggregate and sediment in natural media,[18] a specific exposure design was used to challenge the target organisms with stable concentrations and aggregate size of NPs. This crucial point makes the acquired information easier to compare with other similar studies.[19] Thanks to the ^{47}Ti-labeling and the establishment of a very sensitive quantification procedure with KED ICP-MS, we measured Ti bioaccumulation in mussels exposed at low environmental concentrations. Moreover, we addressed the question of whether ingested TiO_2 NPs are physiologically retained in mussel tissue.

EXPERIMENTAL METHODS

Synthesis of $^{47}TiO_2$ NP. Titanium has five naturally occurring stable isotopes (^{46}Ti, ^{47}Ti, ^{48}Ti, ^{49}Ti, and ^{50}Ti) which can be used for stable isotope tracing methods. The natural abundances of these five isotopes are respectively 8.0%, 7.3%, 73.8%, 5.5%, and 5.4%. The best sensitivities for tracing labeled materials are usually obtained by employing highly enriched isotope with low natural abundance (i.e., ^{46}Ti, ^{47}Ti, ^{49}Ti, and ^{50}Ti). Moreover, the choice of enriched isotope is strongly constrained by the potential interferences occurring in the sample. Even if ICP-MS with KED mode can reduce significantly major interferences, minor interferences can still occur especially at very low Ti concentration. For instance, ^{50}Ti is not a suitable tracer due to interferences generated by ^{36}Ar $+^{14}$N, and ^{49}Ti is not suitable either when a persulfate fusion treatment is performed, because of a serious polyatomic interference from ^{32}S+^{16}O+^{1}H. Once ^{47}Ti was chosen as a tracer, samples contaminated with natural Ti were analyzed in order to assess the best isotope for background determination. Different ratios were verified with ^{47}Ti in the numerator and a nonenriched isotope in the denominator. Deviation from the natural value was attributed to interferences. As described in the Results section, ^{46}Ti appeared to be the most appropriate to measure the background Ti concentration.

Nanoparticles were synthesized following Dufour et al.[20] with minor modifications. $^{47}TiO_2$ powder supplied as a micrometer sized bulk material (1006.6 mg, ^{47}Ti isotopic content 95.7% against 7.44% in natural Ti, Eurisotop, France) was first digested for 6 days at room temperature with 200 mL 12 M hydrochloric acid (HCl) and then heated in a microwave at 200 °C for 2 h after addition of 11.4 g of NH_5F_2. At this stage, we obtained a titanium precursor solution for labeled anatase crystallization. NaOH pellets were added with stirring to the ^{47}Ti solution maintained in an ice bath to precipitate $Ti(OH)_4$. The precipitate was collected by centrifugation (12 000*g*, 15 min), washed three times and resuspended in 100 mL of ultrapure water (MQ), and the pH was adjusted to 6 with HCl. This solution was dispatched into four reactors to proceed to the anatase crystallization using microwave heating (200 °C for 2 h; Synthos 3000, Anton Paar). All $^{47}TiO_2$ NPs were collected by centrifugation and washed four times with MQ water. This $^{47}TiO_2$ NPs batch was used for NP's characterization, digestion assays, and bioaccumulation experiments, while unlabeled TiO_2 NPs were synthesized similarly and used in stability tests. From 1006.6 mg of $^{47}TiO_2$ powder we obtained 140 mg of isotopically labeled NPs (i.e., 14% yield).

Organisms and Exposure to TiO_2 NPs. Zebra mussels (20−24 mm) collected from the Meuse-Marne canal river (France) were acclimated in the laboratory at 16 °C with a 14:10 h light:dark photoperiod for at least 15 days prior to experiments. During this period of acclimation, the mussels were fed daily with 3.2 mg of chlorella powder per mussel[21] and the containers' field water was gradually replaced by moderately hard water (MOD; pH 8; hardness 80−100; U.S. EPA 2002, composition in SI). Cyanobacteria (*Synechocystis* PCC6803) used as the mussel food were precultivated under light at 30 °C,[22] collected by centrifugation (10 000 rpm, 20 min), washed three times and suspended in MOD water.

Experiments were conducted to determine whether TiO_2 NPs labeled with the stable isotope ^{47}Ti could be measured in MOD water and mussel tissue after short exposures at environmentally realistic concentrations. Bioaccumulation experiments included (i) a 1 h direct uptake of $^{47}TiO_2$ NPs from water (ranging from 7 to 120 μg/L); (ii) a 1 h dietary uptake ($^{47}TiO_2$ concentrations from 4 to 830 μg/L and cyanobacterial concentration of 1×10^6 cells/mL, that is, 0.3 to 55.6 mg of $^{47}TiO_2$ per gram of cyanobacteria); and (iii) a 1 h dietary uptake followed by a 72 h depuration period ($^{47}TiO_2$ 100 μg/L and cyanobacteria 1×10^6 cells/mL, that is, 6.9 mg of $^{47}TiO_2$ per gram of cyanobacteria). The experimental setup (using 5 L beakers) is summarized in Supporting Information (SI) in Table S1. The 1 h duration of the NP's uptake is comparable to the food residence times observed in the gut of

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bivalves (i.e., 1−10 h),[23,24] to avoid defecation of NPs and their recycling during the feeding periods. Furthermore, the NP exposure was brief enough to limit the decrease in NP's concentration due to mussel filtration, and long enough to ensure a sufficient Ti accumulation for a subsequent accurate detection. Experiments were conducted under UV-containing light to mimic the light often observed in natural environments (1500 luxes, Repti Glo 5.0; the relative spectral power is reported on SI Figure S1). For every treatment, exposure media (NPs + 5 L of MOD water) were first equilibrated in a polypropylene beaker for 24 h under magnetic agitation at 600 rpm to prevent NP's sedimentation, which could have interfered with their uptake by mussels (see stability study in Results). Then, for each treatment, 10 mussels were transferred on a sieve (1000 μm nylon mesh) hanging in the middle of the beaker. We verified that the activity of zebra mussels was not affected by this agitation (see SI). The pH of the exposure media varied from 8.0 for experiments without cyanobacteria to 8.6 for experiments with cyanobacteria. In experiments with cyanobacterial food for mussels, the pH value was slightly increased by the photoautotrophic metabolic activity of cyanobacteria.[25] This pH range was above the Isoelectric Point of pristine TiO_2 surface,[26] making the surface of the particles negative. The stability was examined in detail using small angle light scattering (SALS).

Water (10 mL) was sampled in platinum crucibles at both the beginning and the end of the 1 h exposure to assess $^{47}TiO_2$ concentrations. After exposure, the 10 mussels were rinsed with MOD water and soft tissues were collected and distributed into five pools of two individuals each. Dietary exposures were performed similarly, except that the mussels were exposed to TiO_2 NPs in the presence of cyanobacteria (1×10^6 cells/mL measured as OD at 580 nm). For depuration, mussels exposed to both $^{47}TiO_2$ NPs and cyanobacteria were rinsed with MOD water and transferred in individual enclosures where they were fed with cyanobacteria, in the absence of NPs, for 72 h. During this depuration time, the cyanobacterial cell concentration, measured as OD at 580 nm, was readjusted at the nominal value six times per day and the feces of each individual mussel were collected in platinum crucibles.

Characterization of $^{47}TiO_2$ NPs and Their Stability in Water Suspension. Transmission electron microscopy (TEM) was coupled with electron diffraction-based spectroscopic tools to characterize pristine $^{47}TiO_2$ NPs (Tecnai spirit G2 apparatus operating at 120 kV). The size distribution of 250 NPs was measured using an image analysis program (ImageJ). The crystalline phase was checked using X-ray diffraction (XRD) (Bruker D8 X-ray diffractometer operating in the Bragg−Brentano reflection mode equipped with a nickel filter to select the Cu−Kα radiation) and the specific surface was calculated from small-angle X-ray scattering (SAXS).[27] NP's stability in MOD water was studied over a 48 h period with absorption ($OD_{330\ nm}$) and small angle light scattering (SALS). This stability test was conducted at 10 mg/L of TiO_2 NPs since it is the lowest concentration allowing a reliable measurement of the gyration radius of the NP's aggregates with our homemade SALS apparatus.[28] The gyration radius of aggregates was obtained from Guinier plots of scattering features, more precisely from the saturation of the intensity at low q-values.[29] In the exposure media, the stability of cyanobacteria and TiO_2 NPs were assessed through $OD_{580\ nm}$ and ICP-MS measurements, respectively. In stability tests, performed with unlabeled NPs, their concentration in exposure medium was set at 350 μg/L to ensure an accurate Ti measurement by ICP-MS. Moreover, imaging by bright field and high angle annular dark field scanning electron transmission microscopy (BF and HAADF-STEM, JEOL 2200FS) were used to confirm that TiO_2 NPs were trapped onto the cyanobacterial mantle of extracellular exopolysaccharides (SI Figure S2, not internalized in the cells), in agreement with our previous observation.[22] SALS measurements were not performed in the presence of cyanobacteria because these food particles corrupt the size measurements of NP's aggregates.

Sample Preparation and ^{47}Ti Analysis by ICP-MS. Among the three techniques that we tested to digest TiO_2 NPs (phosphate and persulfate fusion procedures and an open hot-plate digestion with H_2O_2/HCl/HNO_3), the persulfate fusion method appeared to be the most convenient one for analyzing low concentrations of $^{47}TiO_2$ in water, mussel tissue and feces (see SI) . The persulfate fusion procedure was performed as follows.

We independently collected (i) water samples at both the beginning and the end of the 1 h NP exposure, which were gently evaporated to dryness into platinum crucibles in an oven (90 °C for 12 h); (ii) mussels soft tissues, which were freeze-dried, weighed and transferred into platinum crucibles; and (iii) mussels feces, which were dried at 90 °C. Dried samples were ashed in an oven (to 700 °C at 1 C°/min rate) and 1 g of ammonium persulfate was added before fusion on the flame, using a welding torch. After 10 min, the crucible was cooled and rinsed on a hot plate with HNO_3 (2% v/v) to obtain a 50 mL sample, which was stored in a 50 mL polypropylene tube until analysis. To minimize inadvertent contamination, the platinum crucibles were extensively washed with an extra persulfate fusion, rinsed several times in MQ water and dried prior to reuse.

Water, mussels tissues and feces samples were analyzed by ICP-MS (iCAP Q, Thermo Scientific). The solutions obtained after fusion contain a large quantity of salt that generated interferences on Ti masses. Examples of isobaric and polyatomic interferences that occur on Ti masses are given in SI (SI Table S2). Consequently, analyses were performed including collisional kinetic energy discrimination (KED) that reduced interferences thanks to a low mass cutoff. ^{46}Ti, ^{48}Ti, and ^{50}Ti were also corrected from isobaric interferences measuring ^{44}Ca, ^{52}Cr and ^{51}V. To evaluate the effect of sample matrices on the intensities measured with ICP-MS, we prepared solutions containing various Ti (Inorganic Ventures) and fusion salts concentrations and we tracked anomalies in $^{47}Ti/^{46}Ti$ ratio. The procedural blank was the diluted fusion salt media without Ti. Ti in samples was measured by external calibration using germanium (Ge) for internal standard correction. Each sample was measured four times. The detection limit for ^{46}Ti and ^{47}Ti was 0.0173 μg/L and 0.0086 μg/L respectively.

Calculation of Accumulated ^{47}Ti and Bioaccumulation Parameters. Newly accumulated ^{47}Ti was determined following Croteau et al.[30] The natural background of ^{47}Ti that occurred in each sample was estimated from ^{46}Ti. The net ^{47}Ti uptake ($\Delta[^{47}Ti]$) is by definition:

$$\Delta[^{47}\mathrm{Ti}] = [^{47}\mathrm{Ti}]_{\mathrm{sample}} - [^{47}\mathrm{Ti}]_{\mathrm{background}}$$

Was calculated as follow:

$$\Delta[^{47}\mathrm{Ti}] = p^{47}([T^{47}\mathrm{Ti}] - [T^{46}\mathrm{Ti}])$$

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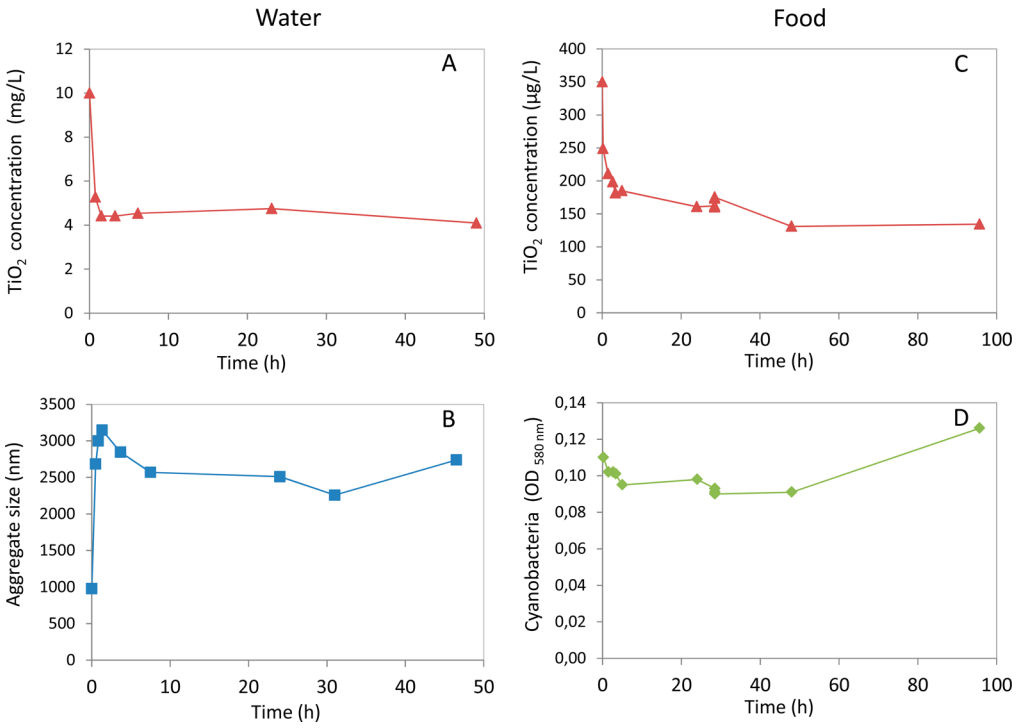

Figure 1. Stability tests performed on the exposure medium comprising MOD water and TiO_2 NPs, in absence (A, B) or presence (C, D) of cyanobacteria *Synechocystis*. TiO_2 concentrations determined by absorption at 330 nm (A) or by ICP-MS (C). Evolution over time of aggregate size determined by SALS (B) and of the cyanobacteria concentration measured by absorption at 580 nm (D).

Where $\Delta[^{47}Ti]_{sample}$ is the concentration of ^{47}Ti in the sample, $[^{47}Ti]_{background}$ is the background concentration of ^{47}Ti that occurred in each sample, p^{47} is the relative abundance of ^{47}Ti, $[T^{47}Ti]$is the total Ti concentration calculated from the tracer's intensity and $[T^{46}Ti]$ is the total Ti concentration calculated from ^{46}Ti's intensity. This methodology allows the determination of potentially elevated and variable Ti natural backgrounds for each individual mussel.

In order to more easily compare the exposure via water to the exposure via diet, the uptake rate constant k_u (L/g/d) was determined by linear regression of ^{47}Ti influx in mussel tissues (μg of $^{47}Ti/g_{mussel}/d$) as a function of exposure concentration (μg of ^{47}Ti/L).

Food ingestion rate IR ($g_{cyanobacteria}/g_{mussel}/d$) was determined from the ^{47}Ti concentration measured in the mussel tissue (μg/g $_{dry\ weight}$), the ^{47}Ti concentration measured in the exposure media (μg of ^{47}Ti/L), the food concentration (g $_{cyanobacteria\ dry\ weight}$/L) and the exposure duration T (d) as follows:

$$IR = \frac{[^{47}Ti]_{mussel} \times [food]}{[^{47}Ti]_{expo} \times T}$$

Assimilation efficiency (AE) was defined as the percentage of ^{47}Ti retained in mussel tissue after 72 h depuration:

$$AE(\%) = \frac{Q_{47_{Ti_{mussel}}}}{Q_{47_{Ti_{mussel}}} + Q_{47_{Ti_{feces}}}} \times 100$$

where $Q_{47_{Ti}}$ is the quantity of ^{47}Ti measured either in the mussel tissue after 72 h depuration or in the feces collected during depuration phase. AE was calculated for individual mussel (n = 8) and then averaged.

Statistics. All data were expressed as mean ± standard deviation. Statistical analyses were performed using XL Stat. The bioaccumulation values in mussels (expressed on a dry weight basis) were compared using Wilcoxon-Mann–Whitney test ($p < 0.05$) and the statistical significance of ^{47}Ti accumulation in mussel over exposure concentrations was assessed using an F test.

RESULTS AND DISCUSSION

Characterization and Stability Testing of TiO_2 NP. To study the effect of environmentally realistic low doses of TiO_2 NPs on the relevant target organism zebra mussels frequently used for biomonitoring, we presently report, for the first time, the synthesis of TiO_2 NPs labeled with the stable isotope ^{47}Ti. The synthesized $^{47}TiO_2$ NPs had a mean size of 10.4 ± 3.3 nm (SI Table S3). Their surface area was 105 m^2/g and their XRD pattern could be assigned to a pure anatase with high crystallization degree (SI Table S3). Very similar characteristics were obtained for the unlabeled TiO_2 NPs that we produced (following an identical synthesis protocol) for control purposes (SI Table S3).

The relevance of the protocol for NP exposure is crucial to meaningfully compare studies and develop predictive models of NP's bioaccumulation. Hence, to measure suitable uptake, bioconcentration factors (BCF), bioaccumulation factors (BAF) or toxicity, NP concentrations need to be accurately determined and stably maintained during NP exposure. Previous studies often underestimated the primordial importance of such verifications. For instance, in the absence of agitation, D'Agata et al.[31] measured a TiO_2 NP concentration of only 82 μg/L in their exposure tanks where in fact, NPs were added at a much higher concentration (10 000 μg/L). Similarly, Johnston et al.[32] could not observe a significant uptake of TiO_2 NPs in fish exposed to a concentration up to 5000 μg/L, probably because the NP concentration in the water of the

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Table 1. Main Exposure Parameters Measured in the Present Study

	exposure concentration			$^{47}Ti/^{46}Ti$ ratio			bioaccumulation parameters			
	values[a]	n[b]	unit	water	mussel	feces	label	values	n[b]	unit
experiment 1	6.8 ± 1.1	2	μg/L of TiO_2	1027.3	5.5		k_u[c]	3.2 ± 0.4	15[d]	$L/g_{mussel}/d$
direct uptake	53.8 ± 4.5	2		68.3	16.2					
	118.5 ± 8.2	2		926.8	44.3					
experiment 2	control				1.2					
dietborne uptake	3.86 ± 0.78	2	μg/L of TiO_2	8.2	1.4		k_u[c]	5.5 ± 0.7[e]	20[d]	$L/g_{mussel}/d$
	15.56 ± 1.53	2		55.4	2.3		IR[f]	0.007−0.063		$g_{cyano}/g_{mussel}/d$
	48.29 ± 2.69	2		860.9	33.4					
	69.40 ± 3.76	2		182.2	12.7					
	271.55 ± 7.15	2		449.7	51.4					
	830.46 ± 41.27	2		281.1	55.0					
experiment 3 elimination after dietborne uptake	108.61 ± 7.53	2	μg/L of TiO_2	110	1.7	48.3	AE[g]	3.0 ± 2.7	8	%

[a]Value obtained from the two measurements performed at the beginning and at the end of the exposure period (mean ± SD). [b]n is the number of samples. [c]k_u is uptake rate constant ($L/g_{mussel}/d$). [d]Number of replicate samples containing two mussels. [e]Value calculated from the linear regression of bioaccumulated ^{47}Ti in mussel tissues vs exposure concentrations for the 4 lowest concentrations (mean ± SE). [f]IR is ingestion rate ($g_{cyano}/g_{mussel}/d$). [g]AE is assimilation efficiency (%) (mean ± SD).

experimental tank decreased over the studied time due to NP's sedimentation.

In the present study, we exposed mussels to $^{47}TiO_2$ under magnetic stirring (600 rpm) to prevent the sedimentation of NPs, which otherwise would have generated a vertical concentration gradient interfering with the reproducibility of the results. We verified that the magnetic stirring of the NPs-suspension in MOD water (the premix) during at least 10 h, stably maintained the concentration and aggregate size of the TiO_2 NPs (Figure 1AB). These TiO_2 NP steady-state aggregates in MOD were modeled as having a radius of gyration of 2500 nm. Similarly, the addition of the cyanobacterial food of mussels did not impair the stability of the agitated NP's premix (Figure 1-CD). Though a 10 h agitation of the premix, with or without cyanobacterial cells, was sufficient for its steady state, we decided to use a slightly longer equilibration time (24 h) for practical convenience. Thus, in the subsequent assays of the NP's effects on mussels, the exposure medium comprising the beaker containing MOD water, the TiO_2 NPs, the nylon sieve basket hanging in the beaker and, when required, the cyanobacterial cells, were first equilibrated for 24 h under magnetic stirring at 600 rpm prior to the gentle introduction of mussels into the nylon basket. This exposure design enabled the TiO_2 NP concentrations to be homogeneous and stable during exposures (the average variation was about 10%; Table 1). Our results showed that stirring does not disturb mussels, which are used to living in moving waters (see the justification in SI). Thus, stirring is better suited to prevent NP's sedimentation[19] than dispersing agents or sonication, which are not environmentally realistic.

Ti Analysis by ICP-MS. The ICP-MS analysis of low metal-concentration samples can be altered by the presence of salts introduced during the NP's fusion step, which can produce interferences on the different isotopes of the analyte (SI Table S2). Hence, we searched for anomalies in $^{46}Ti/^{47}Ti$ and $^{49}Ti/^{47}Ti$ ratios to track interferences and determine the optimal Ti to salt ratios for analyses. In the case of high Ti concentration samples used in the phosphate or persulfate digestion assays described above, ICP-MS measurements were facilitated by the fact that these samples could be highly diluted (200-fold) before analysis. By contrast, for low Ti concentration samples, which could not be diluted that much, salt interferences were observed. The most problematic ones occurred after the phosphate fusion method on ^{47}Ti mass due to $^{31}P + {}^{16}O$, thereby precluding this method to be used for analyzing low ^{47}Ti concentrations. Thus, persulfate fusion was selected and a deeper analysis of interference was realized. As shown in Table 2 interferences had bigger effects on solutions with low Ti concentration and mainly occurred on ^{49}Ti ($^{33}S + {}^{16}O$ and $^{32}S + {}^{17}O$). A S/Ti ratio lower than 100 000 was considered optimal to be confident in the quality of ^{46}Ti and ^{47}Ti measurements. For instance, when S/Ti was 28 543 the $^{46}Ti/^{47}Ti$ ratio obtained, that is, 0.99, was identical to the target values observed in the absence of sulfur salts (0.96−1.02). High sample dilution was favored to minimize chemical damages on sampler and skimmer cones of the ICP-MS due to salts.

Table 2. Natural $^{47}Ti/^{46}Ti$ and $^{47}Ti/^{49}Ti$ Ratios Determined by ICP-MS As a Function of Titanium (μg/L) and Sulfur (mg/L) Concentrations (n = 4)[a]

			$^{47}Ti/^{46}Ti$		$^{47}Ti/^{49}Ti$	
[S] mg/L	[Ti] μg/L	S/Ti	mean	RSD	mean	RSD
0	0.94	0	1.02	12.75	1.11	12.94
0	10.01	0	0.96	2.14	1.03	7.09
0	48.53	0	1.01	1.44	1.07	1.88
28	0.96	29 221	1.04	3.41	0.49	15.84
28	9.50	2951	0.98	1.96	0.96	4.21
28	48.79	575	1.03	3.09	1.06	2.37
280	0.96	292 142	0.72	4.39	0.08	10.01
280	9.83	28 543	0.99	3.11	0.52	3.43
280	44.36	6323	1.00	1.24	0.85	0.84
701	0.96	728 725	0.61	19.58	0.05	8.87
701	8.81	79 543	0.95	2.97	0.32	3.45
701	47.34	14 813	1.02	2.32	0.76	2.40

[a]RSD: relative standard deviation.

Bioaccumulation of $^{47}TiO_2$ NPs in Mussels through Filtration of Waters Containing or Not Their Cyanobacterial Preys. The analysis of Ti bioaccumulation in biological organisms is complicated by the high natural Ti background occurring in their tissues, unless one uses the stable isotope ^{47}Ti to discriminate between the incoming and resident Ti. For instance, Federici et al.[6] could not detect bioaccumulation of

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TiO_2 NPs in rainbow trout exposed at 100, 500, and 1000 μg/L despite a long exposure time (14 days). The Ti concentrations they observed in tissues (5−24 μg Ti/g $_{dry\ weight}$) were similar to the background levels measured in earthworm (9.7 μg Ti/g $_{dry\ weight}$)[5] or in zebra mussels (6.4 μg/g; this study). High $^{47}Ti/^{46}Ti$ ratios were observed in water and mussel tissues, indicating the presence of labeled ^{47}Ti in the two compartments (Table 1). In control experiment, using tissue of mussels not exposed to ^{47}Ti-labeled NPs, the mean of the ratio $^{47}Ti/^{46}Ti$ was reproductively measured at 1.2 ± 0.1, when it ranged from 1.4 to 55 in contaminated mussels. The highest $^{47}Ti/^{46}Ti$ ratios (up to 1000) were observed in the water samples, in agreement with the low Ti background in MOD water. As mentioned above, the natural Ti background concentration in mussels, measured from the isotope ^{46}Ti, was high (6.4 ± 5.1 μg/g), and variable (0.9 to 19.2 μg/g) depending on the individual mussel tested (Figure 2-A).

When mussels were exposed to a low concentration of $^{47}TiO_2$ NPs without their cyanobacterial prey (6.8 μg/L of $^{47}TiO_2$) we observed a ^{47}Ti bioaccumulation of 1.2 μg $^{47}Ti/g_{mussel}$ (Figure 2-B). When mussels were exposed to $^{47}TiO_2$ NP's suspension containing their cyanobacterial prey, the lowest exposure concentration tested was 3.9 μg/L of $^{47}TiO_2$ NPs (i.e., 0.3 mg $^{47}TiO_2/g_{cyanobacteria}$ since cyanobacteria concentration was 14.93 mg/L during exposure) and the resulting bioaccumulation was 0.2 μg of $^{47}Ti/g_{mussel}$ (Figure 2-C). These findings highlight the relevance of the stable isotope-labeling approach to study the accumulation of newly ingested TiO_2 NPs in mussels exposed to environmentally realistic low concentrations (less than 10 μg/L, i.e. similar to what is predicted in European surface waters: 0.4−3.0 μg/L[4]). Furthermore, the stable isotope-labeling protocol allowed us to successfully deal with the high individual variability of Ti background in mussels (Figure 2-A; and other reporter organisms), which cannot be attributable to mussel size variation (SI Figure S3) by applying an individual correction. Thanks to this strategy, we observed an accurate relationship between TiO_2 NP exposure concentration and Ti bioaccumulation (Figure 2B C).

The ^{47}Ti concentration in mussels increased linearly with increasing $^{47}TiO_2$ NP concentration ($p < 0.05$, F test), irrespectively of the presence or absence of their cyanobacterial food, at least for low concentrations (Figure 2). The uptake rate constant k_u from water was 3.2 ± 0.4 L/g_{mussel}/day (r^2= 0.97) and the uptake rate from diet (calculated from the four first points only) was 5.5 ± 0.7 L/g_{mussel}/day (r^2 = 0.97) indicating similar intake rates. We noticed that for exposure concentrations higher than 270 μg/L, ^{47}Ti was accumulated at a lower rate in mussels. This finding is linked to the feeding behavior of mussels. Indeed, the food ingestion rate (IR) decreased from 0.06 to 0.007 $g_{cyanobacteria}/g_{mussel}$/d as the NP concentration increased from 4 to 830 μg/L of $^{47}TiO_2$ in exposure media (SI Figure S4). Though the large variation between individual IR measurements impedes concluding that NP's ingestion triggers a dietary stress to zebra mussels, this notion is supported by previous findings on other organisms.[33,34] Zhu et al.[33] observed a dose-dependent reduction in filtration and ingestion rates of daphnia exposed to 0.1−5 mg/L of TiO_2 NPs. Shoults-Wilson et al.[34] reported that earthworms are able to avoid soil contaminated with Ag NPs. Similarly, damages to digestion and feeding process were also observed in freshwater snails exposed to ZnO NPs.

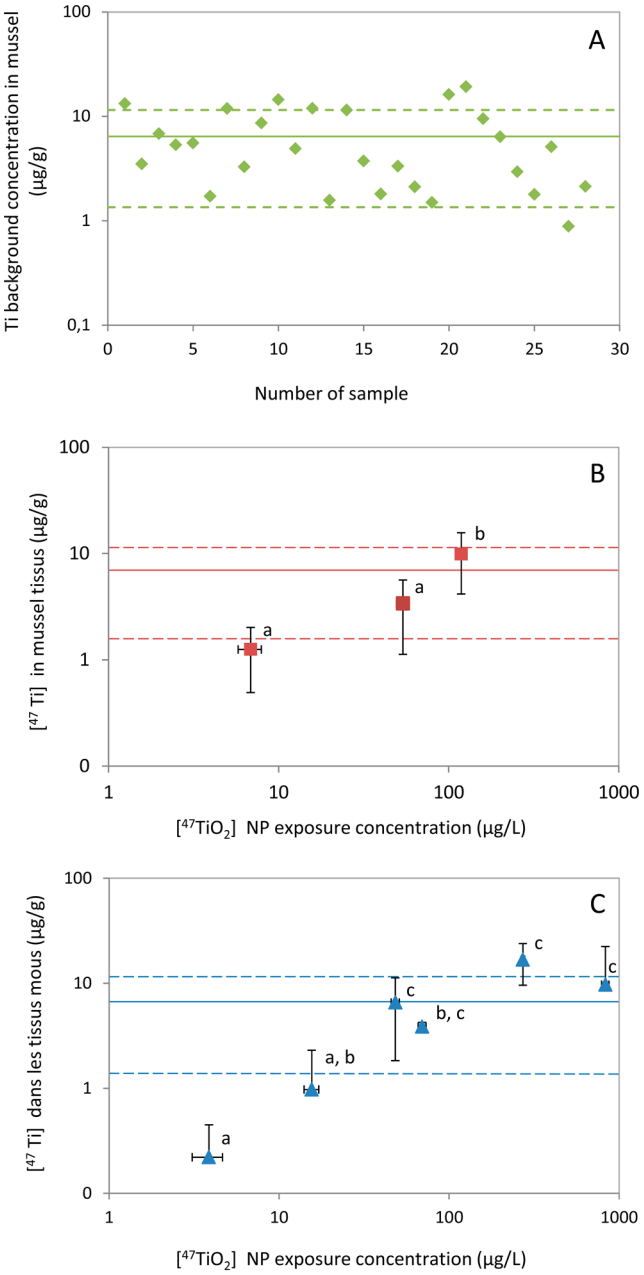

Figure 2. Ti background concentration in mussel tissue (μg/g dry weight) determined from analyses of 28 individual mussels (A) and ^{47}Ti concentrations in mussel (μg $^{47}Ti/g_{dry\ weight}$) exposed for 1 h to $^{47}TiO_2$ NPs in MOD water in absence (B) or presence of 1 × 10^6 c/mL of cyanobacteria as food source (C). The lines display the mean natural background Ti concentrations and the dotted lines show the standard deviation of the mean. Different lower case letters denote a statistical difference among the range of concentration, test Mann−Whitney ($p < 0.05$). .

Elimination of $^{47}TiO_2$ Accumulated from Food. To test whether $^{47}TiO_2$ NPs were accumulated transiently in the gut and/or stably in the internal organs of zebra mussels, we postincubated the contaminated animals in the absence of $^{47}TiO_2$ NPs prior to measuring their ^{47}Ti content. After 72 h of depuration, only a low amount of ^{47}Ti was still present in mussel tissue, indicating that ^{47}Ti elimination is rather easy, probably because of the weak ^{47}Ti biodistribution in internal organs. On the basis of the stable isotope retained after gut

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clearance, assimilation efficiency (AE) was estimated at 3.0 ± 2.7% (mean ± SD, n = 8) suggesting that practically none of the ingested NP is assimilated. The high AE variability can be attributed to interindividual differences between the studied mussels (full set of data are reported in SI Table S4). The very low AE of $^{47}TiO_2$ NPs is similar to what was observed for elements regarded as inert tracers: 0.7−6.2% for Am and 0.2−1.1% for Cr for the mussel *Mytilus edulis*.[35] By contrast, essential metal such as Zn and Cu are usually highly assimilated by animals,[36] since for example the AE of Zn from ZnO NPs reported for a freshwater snail was 86% ± 2%.[13]

Our finding that $^{47}TiO_2$ NPs were transiently accumulated in the gut of mussels, and not much in their internal organs, is consistent with previous reports on other invertebrates, and with what we observed for the unicellular *Synechocystis*.[22] For instance, Zhu et al.[37] showed that for zebra fish fed for 14 days with TiO_2 NPs-contaminated daphnia (at 4.5 mg or 61 mg $TiO_2/g_{daphnia}$), there was no constant increase of Ti accumulation over time, and that the depuration rate was rapid, suggesting that TiO_2 NPs were not internalized. Furthermore, in a terrestrial isopod exposed to 0.1−1 mg of TiO_2/g_{food}, TiO_2 NPs were not internalized,[38] except by digestive gland epithelial cells in animals exposed to higher TiO_2 NPs doses that destabilized the epithelium.[39] One report suggested that rainbow trout could accumulate TiO_2 in their internal organs (liver, brain and spleen), suggesting a NP's transfer across their intestinal epithelium,[40] but this hypothesis was questioned by the data of the same research group a few years later.[41]

The absence of Ti accumulation in internal organs of animals does not indicate that TiO_2 NPs have no detrimental effects on living organisms, since in accumulating at the surface of the cells, TiO_2 NPs can impair the communication of cells with their surrounding medium (respiration, nutrient intake and/or excretion of toxic wastes) or their neighboring cells. Hence, a number of sublethal effects have been reported (i.e., respiratory toxicity, gill pathology such as hyperplasia and edema, oxidative stress, dietary stress, etc.),[6,13] which could result from adsorption of NPs on epithelial surfaces (gill or gut).

There is an interesting paradox between the present observation that most of the ingested NPs can be cleared from the gut of mussels and easily excreted in their feces, and the high natural Ti background that still occurs in the control mussels despite 2 weeks of acclimation in the clean laboratory conditions. Indeed, if TiO_2 elimination is so easy, why is the natural Ti background in mussel not very low after 2 weeks of depuration? It is unlikely that the natural Ti background comes from the bioaccumulation of dissolved Ti associated with a low depuration rate since Ti is mostly in the form of insoluble TiO_2 in the aquatic environment. Thus, it is possible that a small and slow accumulation of TiO_2 can occur in internal organs, and that the excretion of these internalized particles is very inefficient. Similarly, Oberdörster et al.[42] reported that the retention halftime of TiO_2 particles in rat was long because of their difficult excretion (i.e., 117 and 541 days for particles of 250 and 20 nm respectively). The excretion route of internalized TiO_2 NPs remains to be elucidated (the goblet cells have recently been identified as a pathway of excretion[43]) but it is likely that a small fraction of inert TiO_2 NPs will not be eliminated. Whether organisms can or canot excrete TiO_2 NPs accumulated in internal organs is a question requiring further experimentation that will benefit from isotopic labeling, but it raises concerns about the persistent effects of TiO_2 NPs for chronically exposed organisms.

Future Work. By synthesizing TiO_2 NPs labeled with the stable isotope ^{47}Ti we detected bioaccumulation in zebra mussels exposed at environmental concentrations, despite their high and variable natural Ti background. Our results suggest that NP's bioaccumulation simply results from NP's ingestion in the gut, but not internal organs, of mussels. With the stable isotope tracers, it is now possible to meaningfully study the influence of the properties of NPs (shape, size, crystallinity) on their bioaccumulation and possible toxicity. Indeed, our methodology can be adapted easily to the synthesis of NPs with different size, shape and structure. This strategy also gives us the opportunity to combine an accurate measurement of the NP's bioaccumulation on their biological impact on reporter organisms as long as ecotoxicological biomarkers are also measured.

■ ASSOCIATED CONTENT

Supporting Information

Accuracy of digestion techniques, Assessment of a potential TiO_2 NP dissolution, Assessment of stirring on mussel activity, Composition of moderately hard water. Table S1: Experimental setup. Table S2: Interferences occurring on Ti mass. Table S3: NPs characterization. Table S4: AE. Figure S1: Spectral power of the light used during exposures. Figure S2: TEM imaging. Figure S3: Ti background in mussel tissues as a function of mussel dry weight. Figure S4: Ingestion rate. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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■ ABBREVIATIONS

AE	assimilation efficiency
BF	Bright field
HAADF	High angle annular dark field
MOD	moderately hard water
NP	nanoparticle
SALS	Small angle light scattering
SAXS	Small angle X-ray scattering
XRD	X-ray diffraction

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